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Exercise but not mannitol provocation increases urinary Clara cell protein (CC16) in elite swimmers

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Summary
Elite swimmers have an increased risk of developing asthma, and exposure to chloramine is believed to be an important trigger factor. The aim of the present study was to explore pathophysiological mechanisms behind induced bronchoconstriction in swimmers exposed to chloramine, before and after swim exercise provocation as well as mannitol provocation. Urinary Clara cell protein (CC16) was used as a possible marker for epithelial stress.

101 elite aspiring swim athletes were investigated and urinary samples were collected before and 1 h after completed exercise and mannitol challenge. CC16, 11β-prostaglandin (PG)$F_{2\alpha}$ and leukotriene E_4 (LTE_4) were measured.

Urinary levels of CC16 were clearly increased after exercise challenge, while no reaction was seen after mannitol challenge. Similar to CC16, the level of 11β-$PGF_{2\alpha}$ was increased after exercise challenge, but not after mannitol challenge, while LTE_4 was reduced after exercise. There was no significant difference in urinary response between those with a negative compared to positive challenge, but a tendency of increased baseline levels of 11β-$PGF_{2\alpha}$ and LTE_4 in individuals with a positive mannitol challenge.

The uniform increase of CC16 after swim exercise indicates that CC16 is of importance in epithelial stress, and may as such be an important pathogenic factor behind asthma development in swimmers. The changes seen in urinary levels of 11β-$PGF_{2\alpha}$ and LTE_4 indicate a pathophysiological role in both mannitol and exercise challenge.
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Introduction

Elite swimmers have an increased risk for asthma development and the chlorinated pools with exposure to chloramines is believed to be an important pathogenic factor.[1] There is a clear association with duration of exposure to chloramine and degree of hyperresponsiveness and inflammation of the lower airways,[2] as well as asthma prevalence.[3] The mechanism is however still unrevealed. We hypothesise that inspiration of air with high concentration of chloramine may interact with respiratory epithelium in the airways, interfering with mucosal integrity and leading to mucosal barrier dysfunction. This will in turn increase susceptibility to irritative stimuli, and increase the risk of asthma development.

One important factor that is involved in this process is Clara Cell protein (CC16), a small protein secreted from the non-ciliated Clara cells which are found predominantly in the respiratory bronchioles.[4] CC16 is believed to have a protective role against the airway inflammatory response.[4,5] In the airways of asthmatics, fewer CC16-positive epithelial cells have been found compared to controls,[6] and it has been shown that the number of CC16 containing cells in respiratory mucosa correlates with levels found in plasma.[7] Accordingly, lower levels of CC16 in serum from asthmatics children[8] and adults[6,9] have been shown, and there are also studies showing polymorphism in the CC16 gene associated with asthma.[10–12] In context with increased asthma prevalence in swimmers, the link between chloramine and CC16 is plausible. A chronic exposure in swim arenas affect swimming pool attendants to have lower levels of CC16 in serum,[13] indicating some degree of epithelial dysfunction. However, it has been shown that acute chlorine exposure in a swimming pool induce an increase in serum levels of CC16,[14] supporting the hypothesis of CC16 being a protective mediator. In the same way, the levels of CC16 is known to increase in serum after exercise,[15,16] possibly caused by stressing the epithelium through dehydration.

Another effect of this dehydration of the epithelium is the release of inflammatory mediators, such as leukotrienes and prostaglandins that can stimulate the smooth muscle cells to contract. It is well known that primarily cysteinyl-leukotrienes (cys-LT) together with PGD_2 play a critical role in exercise induced bronchoconstriction (EIB), and therapies that block the cys-LT receptor or cys-LT production effectively reduce EIB.[17,18] In addition, it has been shown that some athletes have increased levels of both LTE_4 and 11β-$PGF_{2\alpha}$ in serum and urine after exercise.[19–21]

The diagnosis of asthma in athletes has been under debate during the last years. The use of indirect tests has been advocated as it is believed that indirect provocation tests, in contrast to direct tests like methacholine, better reflects airway inflammation. Another way to clarify the asthma diagnosis could be to explore reactivity related to the actual sport activity. Recently mannitol challenge has been proposed as an alternative indirect test to confirm the presence of exercise induced asthma.[22] Studies on active asthmatics have shown that mannitol reactivity correlates fairly well with other osmotic stimuli as exercise or dry air hyperventilation.[23] Whether the test is suitable as a diagnostic tool in athletes and reflects the same pathophysiological mechanisms is still unknown.

The aim of the present study was to explore pathophysiological mechanisms behind induced bronchoconstriction in swimmers exposed to chloramine, by analyzing urinary levels of CC16, LTE_4 and 11β-$PGF_{2\alpha}$ before and after swim exercise provocation. A secondary aim was to compare these results to provocation with mannitol, as an alternative method resembling the same osmotic provocation as in exercise, in the same environment.

Materials and methods

Subjects

In this study, 101 elite aspiring swim athletes aged 13–23 (mean age 16) years, 55 male and 46 female, who trained a median of 18 (ranging from 10–30) h/week were included (Table 1). At the first occasion they were investigated with skin prick test for air born allergens, exhaled nitric oxide and mannitol provocation. A swim exercise challenge was performed by 97 of these swimmers at a second occasion. The study was approved by the local ethics committee in Lund and all subjects, or parents in the case of subjects who were under age, gave written informed consent.

Skin prick test

A Skin prick test (ALK Abello, Copenhagen, DK) was used to screen for sensitization to a panel of ten common airborne allergens, i.e. pollen (birch, timothy, mug worth), pets (cat, dog, horse), mould (*Claudosporium* and *Alternaria*) and house dust mite (*D. pteronyssinus* and *D. farinae*).

Table 1 Subject characteristics.

	All (n = 101)	Men (n = 55)	Women (n = 46)
Age (years)[a]	16.1	16.2	16
Height (cm)[a]	177	181	170
FEV_1 (% pred)[a]	112	112	112
Doctors diagnosed asthma (%)	36	31	41
Exercise induced symptoms (%)	74	69	80
Atopy (%)[b]	53	51	57
Eczema (%)	52	49	57

[a] Median values.
[b] Defined as a positive skin prick test.

Exhaled nitric oxide

Exhaled nitric oxide was measured by a handheld device (NIOX Mino, Aerocrine, Sweden) according to the ATS/ERS recommendations[24] with an exhaled flow rate of 50 ml/s.

Mannitol challenge

Mannitol challenge was performed in all subjects at site for the sport activities, i.e. in the swimming hall. Mannitol (Aridol™) was inhaled in incremental doses until a drop in FEV_1 of $\geq$15% compared to baseline was reached (defined as a positive challenge) or a maximal cumulative dose of 635 mg delivered (according to manufacturers' protocol, (Pharmaxis, Sydney, Australia)). A flow-volume spirometry (Spira2000, Finland) was performed at baseline and 60 s after each dose. The subjects were observed and a new spirometry was performed after 30 min. The subjects then received inhaled 1000 μg Terbutalin and a new spirometry was performed after 30 min.

Exercise provocation challenge

In the sport specific exercise challenges both males and females were swimming 600 m during 6–8 min. During the first 2 min they exercised targeting a pulse rate of maximum 150 in order to avoid lactate accumulation. Thereafter they finished the lap during the next 4–6 min aiming to achieve a pulse rate $\geq$90% of maximal capacity. Pulse was checked every 100 m during the race by a water proof pulse watch (Polar RS 400). Flow-volume spirometry measuring FEV_1 and FVC (Spira2000, Finland) was performed before the start, immediately after finishing the lap (about 1–2 min after the exercise) and then 5, 10, 15 and 30 min after the race. The subjects then inhaled 1000 μg terbutalin and a new spirometry was performed after 30 min. A 10% fall in FEV_1 after challenge was considered as a positive challenge.

Urine analyses

The urinary samples were collected before and 1 h after completed challenge. CC16 was measured using the Human Clara Cell Protein ELISA kit (detection limit 0.02 ng/ml) from BioVendor (Modrice, Czech Republic) according to the manufacturer protocol, and LTE_4 and 11β-$PGF_{2\alpha}$ were measured using respective EIA kits (detection limits 25 pg/ml and 5.5 pg/ml respectively) from Cayman Chemical (Ann Arbor, MI, USA). Urinary levels were corrected for the amount of creatinine to compensate for dilution. All samples were run in duplicate with an intra-sample variation of <5%.

Statistics

Data is presented as median (25–75 percentiles) and shown in figures as box and whiskers plots. Statistical comparison among the groups was done with Mann–Whitney's *U*-test for unpaired samples and Wilcoxon's test for paired samples. A *p*-value of less than 0.05 was considered significant, and flagged as * = $p < 0.05$ and *** = $p < 0.001$.

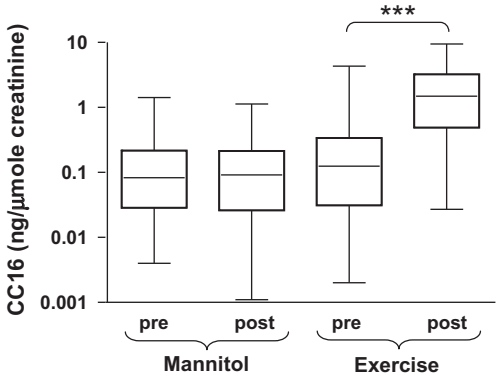

Figure 1 Urinary levels of CC16 in swimmers before (pre) and 1 h after (post) mannitol or swim exercise challenge. *** = $p < 0.001$.

Results

54 subjects were atopic. 14 swimmers had exercise induced bronchoconstrictive response (fall in FEV >10%) and 26 were reactive to mannitol (PD15 $\leq$635 mg). There was no difference in reactivity between atopic and non-atopic subjects (data not shown).

The levels of CC16 were clearly increased ($p < 0.0001$) after exercise challenge (Fig. 1) in 87 out of the 97 samples; from 0.12 (0.031–0.34) to 1.48 (0.49–3.23) ng/micromole creatinine, while no reaction was seen after mannitol challenge; from 0.083 (0.029–0.22) to 0.092 (0.025–0.22) ng/micromole creatinine.

Similar to CC16, the level of 11β-$PGF_{2\alpha}$ was increased ($p = 0.036$) after exercise challenge (Fig. 2); from 49 (32–77) to 57 (38–78), but not after mannitol challenge; from 57 (40–82) to 58 (38–82) pg/micromole creatinine. On the other hand, LTE_4 was significantly reduced ($p = 0.032$) after exercise challenge from 49 (31–83) to 41 (27–70) pg/micromole creatinine, but not after mannitol from 49 (29–70) to 52 (31–82) pg/micromole creatinine (Fig. 3) where however a tendency of an increase in LTE could be seen ($p = 0.065$).

Baseline urinary levels of CC16, LTE_4 and 11β-$PGF_{2\alpha}$ in individuals with a positive compared to a negative

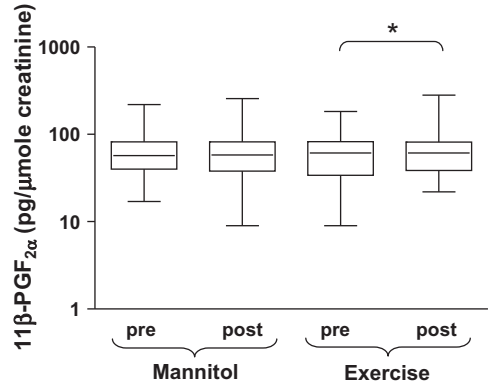

Figure 2 Urinary levels of 11β-$PGF_{2\alpha}$ in swimmers before (pre) and 1 h after (post) mannitol or swim exercise challenge. * = $p < 0.05$.

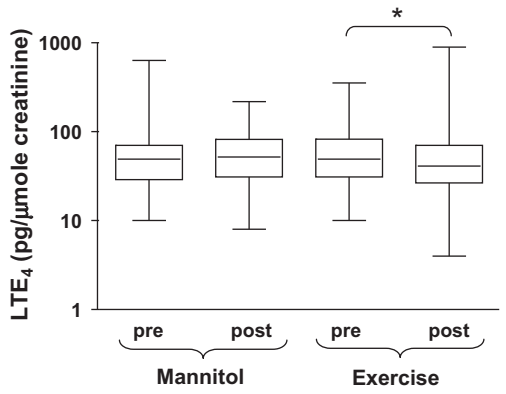

Figure 3 Urinary levels of LTE_4 in swimmers before (pre) and 1 h after (post) mannitol or swim exercise challenge. * = $p < 0.05$.

challenge, were also compared. Interestingly, there was a tendency of increased baseline levels of both LTE_4 ($p = 0.11$) and 11β-$PGF_{2\alpha}$ ($p = 0.14$) in individuals with a positive mannitol challenge compared to a negative, however not significant (data not shown).

There were no differences in urinary CC16, LTE_4 or 11β-$PGF_{2\alpha}$ responses (before versus after) to either mannitol or exercise challenges when comparing individuals with a positive compared to a negative challenge. Neither was there any difference when comparing individuals with or without respiratory symptoms independently of if symptoms were due to exercise or not. Prevalence of doctors diagnosed asthma or exacerbations did not significantly influence either baseline values or differences in urinary levels during either mannitol or exercise challenge, neither were there any direct findings of phenotypic influence, such as atopy or eczema. However, baseline urinary levels of CC16 significantly correlated to exhaled nitric oxide ($p = 0.0095$, $r = 0.26$) (data not shown).

Discussion

The uniform increase of CC16 after swim exercise in pool environment strongly support the idea that exercise interfere with respiratory epithelium in swimmers, and that CC16 could be an important pathogenic factor behind asthma development in swimmers. On the contrary, a similar increase in CC16 was not seen after mannitol challenge showing a remarkable difference concerning involvement of the epithelium in the two tests.

It is believable that CC16 have a protective role in response to epithelial stress. An increase in CC16 levels would then in some unknown way protect the airways from dysfunction due to dehydration of the epithelium. Increased levels of CC16 in serum in response to exercise have been shown previously, but the effects of type of exercise (e.g. breath holding in swimming versus extended heavy breathing in endurance sports) are not defined. It has previously been shown that swimmers have lower levels of CC16 in serum.[13] It is therefore tempting to believe that a tendency of a lower baseline CC16 level in those with a positive osmotic challenge would confirm some degree of acquired epithelial dysfunction, causing an exercise induced bronchoconstriction. In the present study we were unable to see any difference in exercise positive versus negative subject, thus the uniform increase of CC16 most probably reflect some degree of overall signal of epithelial stress. Thus, further studies, i.e. bronchoscopy, are planned to further explore the pathophysiological role of CC16 in asthmatics and swimmers.

Urine levels have been investigated in this paper, and the interactions between the biological and physiological processes that govern transient changes in serum CC16 (i.e. CC16 production and secretion, epithelial permeability and renal clearance) have not been fully characterised, and has to be taken into account. Due to its low molecular weight and small size, CC16 easily diffuses across the bronchoalveolar/capillary barrier and can be detected in peripheral blood serum. Because CC16 is secreted mainly in the respiratory tract, its occurrence in the vascular compartment suggests leakage from the lung into the bloodstream and it is thought to reflect both the rate of synthesis and the permeability of the lung epithelium. Subsequently, CC16 is rapidly cleared from plasma by glomerular filtration. An impaired renal function could of course affect urine concentrations of CC16, but according to Bernard[25] only a large impairment on the glomerular filtration rate affects the levels of CC16, and therefore it is unlikely that this would have any impact on our results. We could nevertheless not exclude that swim exercise give rise to an increased pulmonary epithelial leakiness of CC16 into the bloodstream. However, we assume that an increase in CC16 secretion into the epithelial lining fluid is a more probable mechanism due to previous publications that the serum levels of CC16 correlates with those in bronchoalveolar lavage.[26,27] The renal clearance could however explain the large inter-individual divergences found in the present study. It is also possible that the results would be even clearer if serum would be used when comparing different subject groups.

CC16 is also produced in the male urogenital tract, but previous studies indicate that serum levels are due mainly to pulmonary transudates,[28,29] but is minimized if the first 100 ml urine is discarded.[30] Therefore, the first 100 ml of each male urine collection was discarded in the present study.

The half-life of CC16 in the blood is quite rapid as elevation in serum CC16 induced by swimming was of very short duration, since 2 h after the end of the exercise the serum CC16 had already returned to pre-exercise levels.[15] More precise kinetic analyses show the half-life of CC16 in serum is in fact <18 min.[31] In a recent study, we have found that the increased levels of CC16 in urine is at top about 30–60 min after completed provocation test,[32] hereby urine sampling was done 60 min after completed challenge in this study.

The role of cys-LT and PGD_2 in exercised induced asthma has been shown to be of great importance, showing the significant role of mast cells. Similar to previous publications, this study shows increased levels of 11β-$PGF_{2\alpha}$ after exercise.[21] On the contrary, the levels of LTE_4 were decreased in this study, which are opposite to previous publications.[19–21] This could be explained by difference in phenotype. In the present study, the majority of the subjects had no doctor's diagnosed asthma, but several of those reported asthma symptoms during exercise, in comparison to active atopic asthma in previous

publications. It has also been discussed lately whether a positive provocation test is enough for detecting changes of LTE_4 and 11β-$PGF_{2\alpha}$ in urine, and it has been proposed that the patients would preferably be subjected to further steps in the provocation test to extend the tests and make reliable levels possible (as seen in Ref.[33]). An interesting feature is also that both LTE_4 and 11β-$PGF_{2\alpha}$ have a tendency to predict a positive mannitol test. This further strengthens the hypothesis that mannitol provocation reflects an inflammatory state of the lung. In summary, it is however convincing that both LTE_4 and 11β-$PGF_{2\alpha}$ are involved in bronchoconstriction due to osmotic stimuli.

Exhaled nitric oxide is believed to reflect inflammation in the airways, and its correlation to baseline values of CC16 found in this study indicates that CC16 may have a role in airway inflammation.

In conclusion we show in the present paper that there is a homogenous increase in CC16 in urine after swim exercise. A similar change could not be seen after mannitol challenge, pointing out a clear difference between the tests. The large increase in CC16 after exercise indicates that provocations interfering with the respiratory epithelium may be an important pathophysiological factor. The involvement of CC16 and epithelial dysfunction could also explain the mechanism behind the fact that long time exposure of chloramine increases the risk of asthma development in swimmers. How this relates to other important parts of the inflammatory process in asthma is not clear and thus calls for further studies.

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Conflict of interest

None declared.

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